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SOME CHARACTERISTICS OF PLAQUE VARIANTS OF RIFT VALLEY FEVER VIRUS

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In conducting the research reported here, the investigators adhered to "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

ABSTRACT

Two variants of Rift Valley fever virus have been isolated that differ from each other in plaque size. The biological characteristics of these isolates have been investigated in vivo and in vitro. The size of both variants increased when DEAE dextran was added to the agar overlay. The plaque size of both variants was equally reduced by low concentrations of sodium bicarbonate in the overlay, although the infectivity was not affected. Compared with the large-plaque virus, the small-plaque isolate was more readily adsorbed, more stable at 56 C, grew faster, and had a higher virus yield from monolayers of mouse fibroblast cells. The large-plaque virus required 100 times more plaque-forming units to kill mice by the intraperitoneal route of inoculation than the small-plaque, and also produced a delay in death of the inoculated mice.

I. INTRODUCTION

Rift Valley fever (RVF) virus has been propagated and assayed in a variety of cell cultures.[1] In the plaque assay of this virus, plaques of varying sizes were observed in titrations of virus either before or after passage in mouse fibroblast cell cultures. Two variants were selected, based upon plaque size, and were designated small-plaque (SP) and large-plaque (LP) variants. This report deals with some of the characteristics of these isolates.

II. MATERIALS AND METHODS

A. TISSUE CULTURE

The mouse fibroblast (MFL) cells used in these experiments were similar to the L cell in morphology, karyology, and antigenicity. The medium employed in these cultures was Medium 199[2] containing 10% calf serum.

B. VIRUS

Serum obtained during the viremic phase of RVF virus infection of young lambs, hereafter called infected-lamb serum (LS) virus, was the seed stock. This virus was passed three times in the MFL cells and designated TC. The plaque variants were selected from the assay of this third tissue-culture passage on the basis of size. Each variant was plaque-purified four additional times in MFL cells. The LP and SP viruses were passed a total of five and six times, respectively, in MFL cells.

C. PLAQUE TITRATION

The plaque assay method employed was a modification of that described previously.[3] Two-ounce prescription bottles were inoculated with 5 ml of suspension containing 500,000 MFL cells per ml. After 24 hours, the monolayers were washed and inoculated with 0.1 ml of an appropriate virus dilution. Following a 1-hour adsorption period a primary agar overlay was added consisting of Medium 199, 1% Noble's agar, 20% calf serum, and 0.01% DEAE dextran. Three days later the second agar overlay was added that was identical to the first in composition but also contained 0.007% neutral red. Plaques were visible 1 day later but were enumerated and measured 2 days later.

D. MOUSE ASSAY

The virus titrations in mice (Swiss-Webster, 10 to 14 g) were carried out by the intraperitoneal (IP) or by the intracerebral (IC) routes of inoculation and endpoints were expressed as $MIPLD_{50}$ and $MICLD_{50}$, respectively.

E. TUBE TITRATION

The tube titrations of RVF virus in MFL cells were performed with a serum-free maintenance medium that has been described previously.[3] The endpoints were expressed as the median dose producing a cytopathic effect ($CPED_{50}$).

III. RESULTS AND DISCUSSION

The parent tissue culture virus, TC, produced plaques under agar with diameters varying between 1 and 6 mm. These plaques were visible prior to staining, but only the larger plaques could be enumerated. The plaque variants were picked from the double agar overlay 5 days after the cells were infected. The isolates were selected from each of four additional passages and each continued to breed true. Both the large and small plaques were clear with sharply defined borders.

Both variants exhibited a cytopathic effect in MFL cells as well as in primary chick embryo fibroblast cells. Infected cultures showed cytoplasmic swelling, rounding of the cells with subsequent lysis, and sloughing of the cells from the glass. Homologous antisera prepared in mice and lambs neutralized the corresponding plaque variant as well as antisera prepared against the parent tissue-culture virus. Calf serum known to contain antibodies against RVF virus neutralized both variants.

A. SIZE AND DISTRIBUTION

The size distribution of the variants is shown in Figure 1. The diameters of the plaques of the SP virus ranged from 1 mm to 3 mm, with a mean of 1.5 mm. The plaques produced by the LP virus ranged in diameter from 4 to 7 mm, with a mean of 5.6 mm. Continued incubation at 37 C resulted in increased size. The LP plaques reached 10 to 12 mm 8 days after infection. The SP plaques never enlarged beyond a diameter of 3 mm.

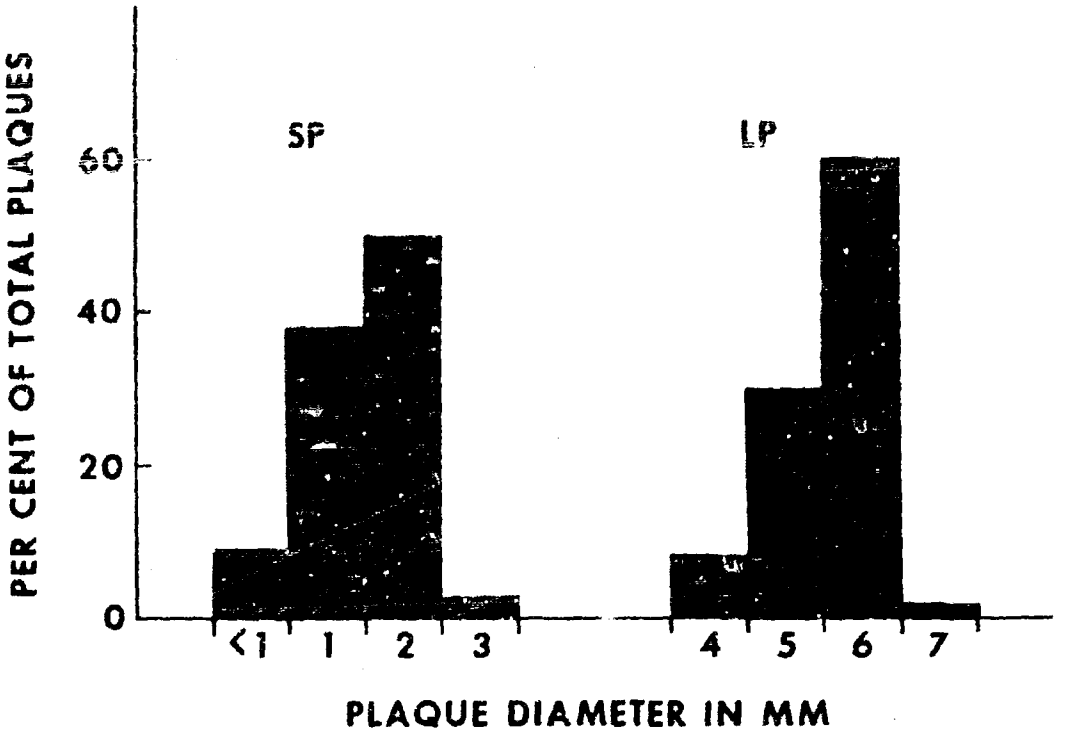

Figure 1. Distribution of Plaque Size for the SP and LP Variants of RVF Virus 5 Days after Inoculation. The mean diameters of the plaques were 1.5 and 5.6 mm, respectively.

B. ADSORPTION

An examination of the adsorption rates of these variants revealed what is probably the most significant factor in explaining the difference in growth rates and yields of virus from infected cell monolayers. Table 1 shows that maximum adsorption of the SP variant occurred within 30 minutes, but only one-third of the LP virus had been adsorbed during the same period. The LP virus required 90 minutes for maximum adsorption, and this longer adsorption period may account for the decreased number of plaques. These findings are similar to those reported by Nagai and Hammon with Japanese B encephalitis virus.[4]

TABLE 1. EFFECT OF ADSORPTION TIME ON THE NUMBER OF PLAQUE VARIANTS OF RIFT VALLEY FEVER VIRUS

Virus	Adsorption Time, minutes	Number of Plaques
Small-Plaque Variant	15	34
	30	42
	60	42
	90	41
	120	40
Large-Plaque Variant	15	8
	30	12
	60	24
	90	37
	120	35

C. EFFECT OF BICARBONATE CONCENTRATION

The influence of varying the concentration of sodium bicarbonate in the agar overlay was examined with each of the variants. Bicarbonate concentrations employed varied from 0 to 2.8 g/liter. The results (Table 2) showed that the plaque size of both variants decreased as the bicarbonate concentration decreased. Although the number of plaques produced by the LP virus was not affected, the number of SP plaques was reduced with diminishing concentrations of bicarbonate. This reduction in the number of SP plaques may have been due to a decrease in plaque size beyond the limits of visibility.

TABLE 2. INFLUENCE OF SODIUM BICARBONATE CONCENTRATION ON SIZE AND TITER OF PLAQUE VARIANTS

Concentration in Overlay, grams/liter	Titer[a]		Average Size, mm	
	Small Plaque	Large Plaque	Small Plaque	Large Plaque
2.8	8.0	6.7	1.5	5.6
1.7	7.8	6.8	1.2	3.6
1.1	7.9	6.8	1.0	3.0
0.3	7.5	6.8	0.5	2.5
0.0	7.6	6.8	0.5	2.0

a. Log_{10} PFU/ml.

D. EFFECT OF AGAR INHIBITOR ON PLAQUE SIZE

Reports earlier[5,6] showed that some mutants of encephalomyocarditis virus and poliovirus were affected by a polysaccharide inhibitor in agar that resulted in the production of small plaques. Later, it was reported that the plaque diameter of one type of western equine encephalitis virus was increased by the addition of diethylaminoethyl (DEAE) dextran to the agar overlay medium.[7]

To determine the effects of the agar polysaccharide on these plaque variants of RVF virus, various concentrations of DEAE dextran were added to the agar overlay. The results of these experiments are shown in Table 3. Both the size and number of plaques produced were reduced in the absence of the dextran. A concentration of at least 100 µg of DEAE dextran per ml of overlay medium was required for maximum size and number of plaques observed with both variants. Unlike some viruses, the large-plaque variant was as sensitive to the inhibitor in agar as was the small-plaque variant, and both variants produced larger plaques with the addition of DEAE dextran.

E. HEAT STABILITY

The heat stability of each variant was examined and compared with that of the parent TC virus. Virus suspensions were prepared in a serum-free maintenance medium[4] with a final pH of 7.6. The suspensions were placed in a 56 C water bath and at various intervals samples were removed and chilled in an ice bath. The samples were assayed by the plaque technique and the results are shown in Figure 2.

TABLE 3. EFFECT OF DEAE DEXTRAN ON THE NUMBER AND SIZE OF PLAQUES FORMED BY VARIANTS OF RVF VIRUS

Virus	Concentration of DEAE Dextran, μg/ml	Size,[a] mm	Plaques[b]
Small Plaque	0	0.5	16
	50	2.0	26
	100	2.5	40
	200	2.5	42
Large Plaque	0	4.0	25
	50	5.0	28
	100	6.0	31
	200	6.0	39

a. Average of approximately 50 plaques.
b. Average number in triplicate samples.

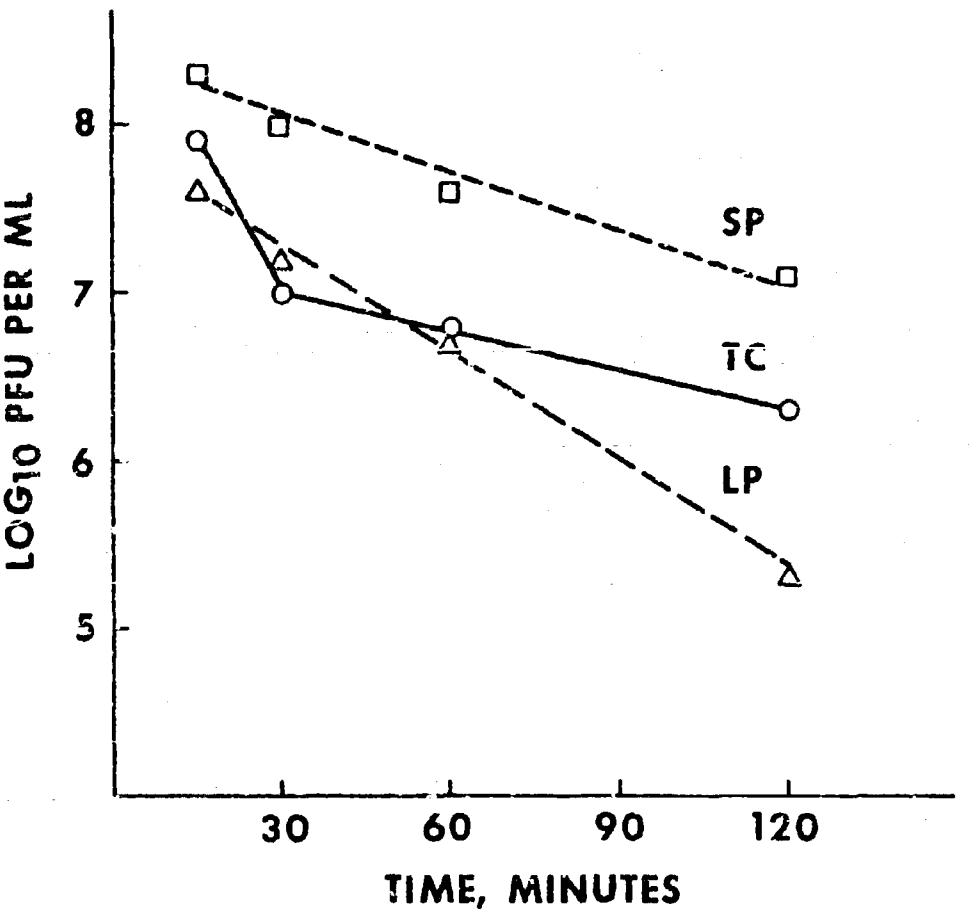

Figure 2. Stability of Parent RVF Virus and the Variants at 56 C.

The stability of the parent TC virus and the SP virus appeared to be comparable after the first 30 minutes. The initial inactivation of the TC virus was rapid, suggesting a heterogeneous population of virus in terms of thermal stability. The rate of inactivation of the LP variant was greater than that of the others tested but the inactivation curve suggested a rather homogeneous population.

F. GROWTH OF VARIANTS

Growth of each variant was studied by inoculating MFL cells with a multiplicity of 10. The virus was allowed to adsorb for 2 hours at 37 C, after which fresh medium was added. At 2-hour intervals infected cultures were removed, frozen and thawed twice prior to plaque assay. These results are shown in Figure 3.

Both variants had a 6- to 8-hour lag period. The SP virus replicated more rapidly and yielded a higher final titer, probably because of its greater efficiency of adsorption. The reduced adsorption rate and thermal lability of the unadsorbed LP virus undoubtedly resulted in a lower effective inoculum multiplicity of this virus as compared with the SP virus.

G. INFECTIVITY

The SP and LP variants were compared with the TC parent and LS viruses by intraperitoneal (IP) and intracerebral (IC) inoculation of mice and by their capacity to produce a cpe and form plaques on MFL cell monolayers. The relative infectivity of the four viruses is shown in Table 4 as ratios of the PFU endpoint to the mouse tests or cpe titers. By IP inoculation of mice the LS virus was the most virulent and the LP virus was the least virulent. By that test the SP virus was 100-fold higher in virulence than the LP variant. Similar findings were seen by IC inoculation of mice; however, the SP virus showed only a 50-fold increase in virulence compared with the LP virus. All four strains were comparable in their ability to cause a cpe in tube cultures of MFL cells.

Further evidence for the reduced virulence of the LP virus was shown in the delay of death of infected mice. To eliminate the possibility that variable doses might invalidate the comparisons, the variants and TC virus were appropriately diluted so that each mouse inoculated received 200 $MIPLD_{50}$ doses by the IP route. The number of deaths occurring on each day were recorded and are shown in Table 5. Similar results have been obtained repeatedly with different cell passages of the LP virus.

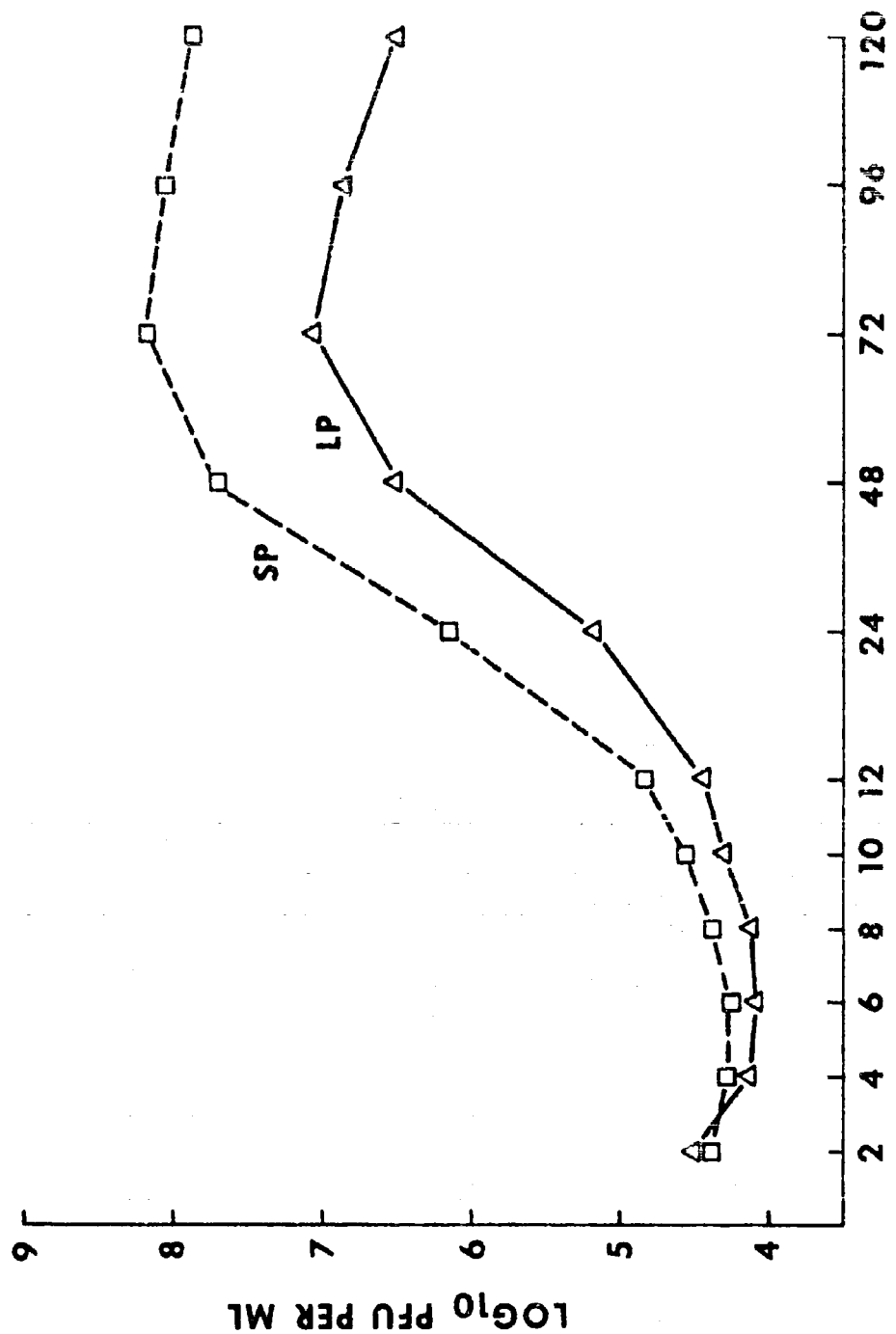

Figure 3. Growth of the Variants of RVF Virus in MFL Cells.

TABLE 4. RELATIVE INFECTIVITY OF THE PARENT (TC) AND PLAQUE VARIANTS OF RIFT VALLEY FEVER VIRUS

Titer Ratio	Virus			
	SP	LP	LS	TC
PFU	8.4	7.2	9.1	7.8
PFU/MIPLD$_{50}$	8	800	0.08	8
PFU/MICLD$_{50}$	0.5	25	0.02	6
PFU/CPED$_{50}$	1.2	1.2	1.0	1.2

TABLE 5. CUMULATIVE MOUSE DEATHS OBSERVED WITH THE PARENT (TC) AND PLAQUE VARIANTS OF RIFT VALLEY FEVER VIRUS[a/]

Days Postinfection[b/]	Virus		
	TC	LP	SP
1	0	0	0
2	7	0	10
3	24	6	27
4	30	23	32
5	31	30	32
6	31	32	32
7	31	32	32
mean day of deaths	3.0	4.2	2.8

a. 200 MIPLD$_{50}$ doses.
b. Inoculated by intraperitoneal route.

Although these variants were isolated only on the basis of their difference in plaque size, these results suggest that the isolates are true variants of RVF virus and not the result of components such as inhibitors that are in the test systems. Furthermore, unlike previous reports[8] of the reduction of plaque size associated with attenuation of some viruses, the small-plaque isolate of RVF virus was more virulent than the large-plaque isolate.

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